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⑤④ **Liposome/anthraquinone drug composition and method.**

⑤⑦ A drug/liposome composition comprises an aqueous suspension of liposomes and, entrapped in the lipid bilayer region of the liposomes, an anthraquinone drug containing quinone and hydroquinone functionalities on adjacent anthracene rings. An iron-specific trihydroxamic chelating agent contained in the bulk aqueous phase of the suspension and a lipophilic free-radical scavenger contained in the bilayer region of the liposome cooperate to reduce chemical modification of both drug and lipid components of the composition. The compositions can be used for administering doxorubicin intravenously to humans, with significantly reduced incidence of side effects, and for treating primary and secondary neoplasms of the liver.

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LIPOSOME/ANTHRAQUINONE DRUG COMPOSITION AND METHOD

1. Field of the Invention

The present invention relates to an improved liposome/anthraquinone therapeutic composition having reduced lipid oxidation and free-radical damage, to methods for preparing and using the composition.

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3. Background of the Invention

Liposome delivery systems have been proposed for a variety of drugs, particularly those which are administered parenterally. Liposomes have the potential of providing a controlled "depot" release of the administered drug over an extended time period, and of reducing toxic side effects of the drug, by limiting the concentration of free drug in the bloodstream. Liposomes can also alter the tissue distribution and uptake of drugs, and the altered tissue distribution, combined with other advantages just mentioned, can significantly increase the therapeutic effectiveness of the drug. Liposome/drug compositions can also increase the convenience of therapy by allowing higher drug dosage and less frequent drug administration.

Liposome drug delivery systems are reviewed generally in Poznansky et al.

One group of drugs whose use in liposome delivery systems has been widely studied is the class of anthracene quinones, including, particularly, the anthracycline glycoside antibiotics, exemplified by the anti-tumor drug doxorubicin or "Adriamycin", doxorubicinol, daunorubicin, and daunorubicinol, and their cyanomorpholinyl derivatives. Doxorubicin (DXR) is a potent chemotherapeutic agent effective against a broad spectrum of neoplasms (Aubel-Sadron et al and Young). However, use of the drug in soluble form is limited by serious side effects. Its acute toxicity in humans includes malaise, nausea, vomiting myelosuppression, and severe alopecia. In addition, cumulative and irreversible cardiac damage occurs with repeated administration, which seriously limits the use of the drug in protracted treatment (Young).

Previous animal model studies indicate that when DXR is administered in liposome form, the drug retains its therapeutic effectiveness against animal tumors, but is significantly less toxic, as judged by

reduced mortality (Forssen, Gabizon 1985). The drug-protective effect of liposome may be due, at least in part, to an alteration in tissue disposition and drug-release rate of the injected drug (Gabizon 1982; Gabizon 1983; Juliano).

The cardiomyopathy observed in DXR treatment is similar to the cardiac muscle lesions seen in experimental animals under conditions of alpha-tocopherol (α -T) deficiency (Tomasz), suggesting that the drug-induced lesions are caused by increased free-radical reactions involving membrane lipids. DXR and other anthraquinones, which have the general structure shown in Figure 1, contain both quinone and hydroquinone groups, and thus might be expected to promote peroxidation reactions involving electron transfer to or from the quinones or hydroquinones. In addition, binding of the drug to lipids through the anthracene moiety would be expected to facilitate lipid involvement in peroxidations reactions. It is known, for example, that DXR binds tightly to cardiolipin, a major lipid component in mitochondria, and enzyme-catalyzed electron transport results in formation of covalent linkages between the drug and lipid (Goormaghtigh).

The free radical mechanism of cardiac toxicity proposed for DXR suggests that a lipophilic free-radical quencher such as vitamin E would be effective in reducing drug toxicity and this, in fact has been found (Myers; Wang; Sonneveld). The referenced animal studies have shown that vitamin E is effective in reducing cardiotoxicity when administered prior to or concurrent with DXR administration. More recent studies have shown that a liposome drug system with coentrapped DXR and vitamin E is less toxic in mice, and produces less cardiomyopathy, than either vitamin E/DXR or liposome/DXR combinations alone (Olson). The reduced toxicity of liposomes with coentrapped DXR and vitamin E apparently results from a combination of the altered drug distribution and/or lower free drug levels --due to liposomal entrapment of the drug --and from reduced free-radical damage --due to the free-radical quenching activity of vitamin E.

In summary, animal studies performed to date indicate that liposomes can reduce the cardiotoxicity of DXR and that the presence of vitamin E in the liposomes may further reduce cardiotoxic side effects. The studies do not suggest, however, whether liposomes have the ability to reduce more short-term and generalized toxicity side effects seen in humans, such as nausea, malaise, hair loss, and bone marrow depression, which are routinely associated with DXR treatment. Here it is noted some of the side effects of DXR administration, particularly nausea, can be ameliorated by slow-drip drug intravenous administration, typically over a period of 2-4 days, as has been described in the prior art. In this method, the patient is prepared by central vein catheterization, and equipped with a refillable pump that is used to deliver the selected dose over a total dosing period of typically several days. This method has not been widely adopted because of the inconvenience and logistical problems involved. Nor does the method produce a significant reduction in the incidence of alopecia.

Additionally, the animal studies conducted heretofore have not examined nor appreciated the extent and effect of lipid and drug oxidation which may occur on storage of drug/liposome formulations. It has been found, in studies conducted in support of the present invention, that the drug and lipids in such formulation can undergo substantial chemical modification on storage, even under anoxic storage conditions. Such damage increases the toxicity of the drug formulation and appears to compromise the therapeutic action of the drug.

4. Summary of the Invention

The present invention includes a liposome/anthraquinone composition having anti-oxidant properties designed to reduce lipid oxidation and free radical damage to both the lipid and drug components of the composition.

According to the invention, it has been discovered that drug and lipid damage related to oxidative and free-radical mechanisms is substantially reduced by including in the liposome formulation, a lipophilic free-radical scavenger, such as α -T, and a water-soluble trihydroxamic acid chelating agent, such as ferrioxamine, having a high and selective binding affinity for ferric iron. The extent of protection against lipid-peroxidation and free-radical damage is much greater than that afforded by free-radical quenchers alone or by chelating agents, such as ethylenediamine tetraacetic acid (EDTA), which have been used heretofore. The chelating agent is present in a molar excess of the ferric iron in the suspension.

One preferred liposome composition contains DXR (or a pharmacologically acceptable analog, derivative, or salt thereof) at a molar ratio of at least about 2.5 mole percent in liposomes composed 20-50 mole percent cholesterol, 10-40 mole percent negatively charged phospholipid, such as phosphatidylglycerol (PG), phosphatidylserine (PS) or phosphatidylinositol (PI), and phosphatidylcholine (PC). The liposomes are predominantly in the size range of 0.05 to 0.5 microns, and preferably between about 0.05-0.25 microns.

and the amount of free drug in the suspension --that is, drug not associated with liposomes --is preferably less than about 15% of the total drug in the suspension. Ferrioxamine is contained in the suspension at about 50 μ M, and α -T in the liposomes, at a concentration of at least about 0.2 mole percent.

Also included in the invention is a method for treating human neoplasms with a DXR/liposome composition, preferably the composition as formed above. Clinical trials on human cancer patients in the frame of a Phase I study indicate significant reduction of many side effects such as discomfort (malaise), headaches, nausea, vomiting, local pain at the site of injection, and alopecia, frequently associated with treatment by free DXR. Therapeutic effectiveness against human primary and secondary neoplasms of the liver have been demonstrated in cases patients who have failed or ceased to respond to other forms of therapy, including, in some cases, therapy by free doxorubicin.

More generally, this aspect of the invention includes a method for significantly reducing nausea and alopecia with adriamycin administration, by administering the adriamycin, at therapeutically effective doses, in liposome-entrapped form, and a method for treating primary and secondary neoplasms of the liver in humans, by administration of DXR in liposomal form to the bloodstream.

It is one general object of the invention to provide a liposome composition containing an entrapped anthraquinone drug in which in vitro peroxidative damage to the lipid and drug components of the composition is substantially reduced.

Another object of the invention is to provide a general method for reducing, in a liposome/anthraquinone composition, lipid and drug toxicity which are related to oxidation and free-radical reactions.

A specific object is to provide such a composition for treatment of human neoplasm, wherein the drug is DXR or a pharmacologically accepted analogue, derivative, or salt thereof.

A related object of the invention is to provide a method of treating human neoplasms, and, particularly, primary and metastatic liver tumors, hematopoietic proliferative disorders, and leukemias with liposome-entrapped DXR with significant amelioration of normal drug side effects.

These and other objects and features of the invention will become more fully apparent when the following detailed description of the invention is read in conjunction with the accompanying drawings.

Brief Description of the Drawings

Figures 1A-1C shown the general structures of class I and II anthracycline aminoglycosides (1A and 1B, respectively), and mitoxanthrone (1C);

Figure 2 shows the fluorescent emission spectra of DXR in soluble (upper dashed line) and liposome-bound (upper solid line) form, and DXR-Fe (III) complex in soluble (lower dashed line) and freshly prepared liposome-bound (lower solid line) form;

Figure 3 illustrates oxidative and free radical mechanisms which may contribute to drug and lipid modifications in the presence of ferric iron, oxygen, and unsaturated lipids;

Figure 4 illustrates ferric iron chelation by a trihydroxamic acid chelating agent;

Figure 5 shows UV spectra of lipids isolated from liposomes incubated in the presence (spectrum A) or absence (spectrum B) of ferrioxamine;

Figure 6 plots the effects on survival in mice of graded doses of free DXR (F-DXR) and liposome-entrapped DXR (L-DXR) after a single injection;

Figures 7 and 8 are plots of the survival curves for male and female mice, respectively, after repeated administration of graded doses of F-DXR and L-DXR; and

Figure 9 is plot showing plasma molar concentration of adriamycin, as a function of hours following administration of free drug (solid lines) and liposome-entrapped drug (dashed lines).

Detailed Description of the Invention

I. Preparing the Liposome/Drug Composition

A. The Anthraquinone Drug

The anthraquinone drug used in preparing the composition of the invention is an anti-neoplastic anthraquinone drug having an anthracene ring structure and quinone, semi-quinone, or hydroquinone functionalities carried on the ring structure. More precisely, and as the term is defined herein, anthraquinone drugs include those anthraquinone structures having one quinone and hydroquinone group on adjacent

rings of the anthracene ring structure. Anti-neoplastic anthraquinones having these features can be classed into three groups which are illustrated in Figures 1A-1C.

The first group is the class I anthracene glycosides which are illustrated in Figure 1A. Included in this group are a number of clinically important anti-neoplastic drugs, such as doxorubicin (DXR, commonly known as Adriamycin), daunomycin, carinomycin, N-acetyladiamycin, N-acetyldaunomycin, rubidazole, and 5-imidodaunomycin. Table I below gives the structural variations of these several class I drugs, in terms of the R₁, R₂, and R₃ groups in Figure 1A. More recently, a cyanomorpholino derivative of DXR has been reported (Sikic). Drugs in this class are known to have anti-neoplastic effects against a variety of cancers, including acute leukemias, breast cancer, Hodgkin disease, non-Hodgkin lymphomas, and sarcomas. The primary mechanism of the drugs appears to be intercalation into DNA and DNA damage (Young, Goormaghtigh 1984), although membrane binding (Goormaghtigh 1984) and enzyme-catalyzed free-radical formation (Aubel-Sadron) have also been suggested as possible contributing mechanisms of drug action.

TABLE I

	R ₁	R ₂	R ₃
Adriamycin	=O	-CO-CH ₂ OH	-NH ₂
Daunomycin	=O	-CO-CH ₃	-NH ₂
N-Acetyladiamycin	=O	-CO-CH ₂ OH	-NH-CO-CH ₃
N-Acetyldaunomycin	=O	-CO-CH ₃	-NH-CO-CH ₃
Rubidazole	=O	-C-N-NH-C- CH ₃ O	-NH ₂
5-Imidodaunomycin	=NH	-CO-CH ₃	-NH ₂

The second group includes the class II anthracene glycosides, which are distinguished from the class I compounds by more complex (multimeric) aminoglycoside residues, as seen in Figure 1B. These compounds share the same general therapeutic and toxicity properties of their class I counterparts. Representative class II anthracene aminoglycosides are listed in Table II, with reference to R₁, R₂, and R₃ groups shown in Figure 1B.

TABLE II

	R ₁	R ₂	R ₃
Anthracycline			
Musettamycin	OH	COOCH ₃	H
Rudolfomycin	OH	COOCH ₃	Rednosamine
Aclacinomycin	H	COOCH ₃	Cinerulose
Marcellomycin	OH	COOCH ₃	2-Deoxyfucose
Descarbomethoxy-marcellomycin	OH	H	2-Deoxyfucose
Descarbomethoxy-rudolfomycin	OH	H	Rednosamine

The third group of anthraquinones includes those which have the general ring structure shown in Figure 1A, i.e., with quinone and hydroquinone groups on adjacent anthracene rings, but which lack the glycoside groups characteristic of the anthracycline antibiotics. One important drug in this group is mitoxanthrone, an anti-neoplastic drug which is reported to be effective against a variety of cancers, including breast cancer, but which may have reduced side effects, including cardiotoxicity, when compared with free DXR (Stuart-Harris).

Studies on the binding of ferric iron to DXR which have been carried out in collaboration with one of the inventors indicate that the anthraquinone drugs, and particularly the anthracycline aminoglycosides, form stable metal complexes with ferric iron (Samuni). The studies examined the binding of DXR to ferric iron, both in the presence and absence of small unilamellar vesicles (SUVs) formed from the saturated phospholipid dimyristylphosphatidylcholine (DMPC). With reference to Figure 2, the fluorescent emission spectrum of DXR at 480 nm excitation wavelength (upper dashed line) shows peaks at about 590 and 560 nm, and this spectrum is changed very little when the drug is associated with SUVs (upper solid line). When DXR is complexed with ferric iron in the presence of ADP, a fluorescence emission spectrum having nearly the same shape but significantly reduced intensity was observed (lower dashed line). The reduced intensity presumably results from the quenching effect of iron. When the DXR-Fe(III) complex was added to DMPC SUVs, less quenching effect was observed, as evidenced by a fluorescence emission spectrum which was intermediate in intensity between that of the free drug and the DXR-Fe(III) complex in solution (lower solid line). The same emission spectra for DXR-Fe(III) complex in association with liposomes was observed whether iron was added before or after drug binding to the liposomes, indicating that the drug-iron complex forms readily with lipid-bound drug.

Of interest with respect to the present invention was the finding that DXR undergoes relatively rapid and irreversible chemical modification when complexed with ferric iron in the presence of SUVs formed with saturated and/or unsaturated phospholipids. One measure of this modification is a change in the 590/560 nm ratio of the fluorescence spectra, which would result from modification of the anthracene ring. The 590/560 nm ratio of DXR-Fe(III) in association with DMPC is initially about 1.9. In the presence of ferric iron, at an iron:drug ratio of 2:1, this ratio falls to about 0.9 over a five hour period at 30°C. With a large molar excess of ferric iron, the decline in 590/560 is significantly faster, decreasing to less than 0.8 in two hours. The modified DXR, when extracted from the liposomes, has a 590/560 ratio of about 0.6. The modified drug also shows increased hydrophobicity, as evidenced by its partitioning characteristics in a standard biphasic partition system. This change may be due to cleavage of the more hydrophilic sugar moiety from the anthracene ring structure. It is noted that the drug modification reactions occur either in the presence or absence of oxygen, in liposomes composed either of saturated or unsaturated phospholipids. That is, the events leading to drug modification do not appear to require oxygen dependent electron transfer or free radical formation involving unsaturated lipids. However, as will be seen below, both oxygen and unsaturated lipids do contribute to drug modification in the drug/liposome formulation, and under these conditions, significant lipid peroxidative damage may also occur. One of the important aspects of the formulation of the invention, as will be seen below, is a significant reduction in such drug and lipid modifications.

B. Lipid Components

The liposomes in the composition are formed from standard vesicle-forming lipids, which generally include neutral and negatively charged phospholipids and a sterol, such as cholesterol. The selection of lipids is guided by considerations of (a) drug-release rate in serum, (b) drug-entrapment efficiency, (c) liposome toxicity, and (d) biodistribution and targeting properties.

Considering the effect of lipid components on in vivo drug-release rates, the most important compositional factors are chain length, degree of unsaturation, and head group charge and side groups in the phospholipids. The dependence of drug release rate on lipid composition is due in part to differences in the rate of exchange of amphipathic drug with the outer liposome bilayer, and in part to the differing stability of liposomes having different lipid compositions. From studies below, it will be seen that negatively charged phospholipids, such as phosphatidylglycerol (PG) and phosphatidylserine (PS), tend to enhance drug liposome stability as measured by DXR release in 50% plasma, whereas cardiolipin (CL) produces a marked destabilizing effect on the liposomes. The latter effect may be related to the apparently strong interaction between DXR and cardiolipin which is reflected by drug and lipid cross-linking which also occurs in vivo (Goormaghtigh 1983). Neutral phospholipids, particularly phosphatidylcholine (PC), and negatively charged phospholipids, such as PG, PS, and phosphatidylinositol (PI), having acyl chain components of selected chain lengths and degree of saturation are available from commercial sources, or can be prepared

by known techniques.

Another lipid component which is important to liposome stability, and therefore to drug-release rate, is cholesterol. In one study conducted in support of the present invention, the *in vitro* release of DXR from liposomes whose lipid components contained varying amounts of cholesterol, PC, and PG, PS, or CL, was examined. The liposomes were prepared substantially as described in Example I below, but contained the mole percentages of PC, PG, and cholesterol indicated in Table III below. Prior to each a test, the liposome suspensions were freed of free (soluble) drug by molecular sieve chromatography. The DXR/liposome compositions were each incubated at 37°C for 1 hour in the presence of 50% human plasma, and the liposome-associated drug then separated from released, soluble drug, either by molecular sieve chromatography or ultracentrifugation. The amount of DXR retained in the liposomes was calculated as a percentage of the total original drug. The results are shown in Table III:

TABLE III

		% Liposome-Retained	
Liposome Composition		Mole Ratio	DXR
20	PC:CHOL (DXR)	4:4	72
	PC:CHOL (DXR)	4:2	62
	PC:CHOL (DXR)	4:1	76
	PC:CHOL (DXR)	4:0	29
25	PG:PC:CHOL (DXR)	3:7:5	99
	PG:PC:CHOL (DXR)	3:7:4	96
	PG:PC:CHOL (DXR)	3:7:2.5	81
	PG:PC:CHOL (DXR)	3:7:0	70
30	PS:PC:CHOL (DXR)	3:7:10	98
	PS:PC:CHOL (DXR)	3:7:0	62
35	CL:PC:CHOL (DXR)	1:4:5	39
	CL:PC:CHOL (DXR)	1:4:0	28

As seen from the table, 20 mole percent cholesterol produces a 2-fold to 3-fold enhancement in drug retention in PC liposomes, although no additional improvement is seen up to a cholesterol mole ratio of 50%. In liposomes formed with 30 mole percent PG, good drug retention achieved in the absence of cholesterol, but progressively greater stability is observed with increasing amounts of cholesterol. PS, another negatively charged phospholipid, gives substantially the same result as found with PG. Additional studies not reported here indicate that the increased drug retention is seen over a range of negatively charged phospholipid of between about 10-40 mole percent. Interestingly, the presence of only 10 mole percent cardiolipin (diphosphatidylglycerol), which contains a double negatively charged head group, substantially eliminated the cholesterol effect, giving poor drug retention even at 50 mole percent cholesterol.

Drug-entrapment efficiency, another factor to be considered in selecting a lipid composition, refers to the total amount of drug which can be loaded into liposomes, expressed as a ratio of drug per mole per liposome lipid. High entrapment efficiency is desirable both in terms of preparation costs and for maximizing the amount of drug which can be delivered in liposomal form in a give volume of liposomes. Experiments conducted in support of the present invention have examined the efficiency of DXR entrapment in both large and smaller, sonicated liposomes, and with a number of different lipid compositions. The liposome and lipid composition variables are shown in Table IV below. In this table, MLV refers to large multilamellar vesicles, which typically contain heterogeneous liposome sizes averaging between about 0.5-5 microns, and SUVs, to sonicated but unfractionated vesicles. The final entry is for vesicles which have been sonicated and fractionated to remove larger vesicles, yielding predominantly small liposomes which are designated SUV(F). DPPG and DPPC are abbreviations for dipalmitoyl PG and dipalmitoyl PC, respectively.

as distinguished from PC and PG, which are derived from egg lipids and which contain a mixture of both saturated and unsaturated phospholipid components.

TABLE IV

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	Liposome Composition	Mole Ratio	DXR Entrapment	
			% Total	DXR/Phospholipids m mole/mole
10	MLV-PC:CHOL:DXR	4:4:1	14	35
	MLV-PC:DXR	4:1	10	26
	MLV-CL:PC:CHOL:DXR	1:4:5:1	64	128
15	MLV-CL:PC:DXR	1:4:1	58	116
	MLV-PS:PC:CHOL:DXR	3:7:10:2	59	129
	SUV-PC:CHOL:DXR	10:10:1	15	16
20	SUV-CL:PC:CHOL:DXR	1:4:2:1	47	94
	SUV-CL:PC:CHOL:DXR	1:4:5:1	45	90
	SUV-CL:CHOL:DXR	10:5:2	90	180
	SUV-PS:PC:CHOL:DXR	3:7:4:2	50	100
25	SUV-PG:PC:CHOL:DXR	3:7:4:2	61	128
	SUV-DPPG:PC:CHOL:DXR	3:7:4:2	39	78
	SUV-DPPG:DPPC:CHOL:DXR	3:7:4:2	28	56
30	SUV(F)-PS:PC:CHOL:DXR	3:7:4:2	25	50

The data relating to MLVs suggest that a negatively charged phospholipid --either CL or PS --is required for good entrapment efficiency, but that cholesterol has only a minor effect, if any, on drug entrapment. With SUVs, a similar increase in entrapment efficiency was observed with either CL or PG, or PS. Also, entrapment efficiency was relatively poor when saturated phospholipids (DPPG alone or with DPPC) were substituted for the more unsaturated egg lipids, and in the predominantly very small SUV(F) liposomes. The data in Tables III and IV, taken together, suggest that the optimal liposome composition, for obtaining both high entrapment efficiency and good drug retention *in vitro* (in serum plasma), contains between 10-40 mole percent negatively charged phospholipid, particularly PG, but excluding cardiolipin (CL); between 20-50 more percent cholesterol; and at least about 40 mole percent of a natural phospholipid, such as PC. Within this range of lipid components, the degree of acyl chain saturation and chain length may be varied to achieve desired drug-release characteristics *in vivo*, although it would be expected that a composition containing a high percentage of saturated lipids would have a reduced drug entrapment efficiency.

Another important consideration in the choice of lipid components is the toxicity of the drug/liposome composition which is produced. Since the soluble drug is generally more toxic than liposome-entrapped drug, it is desirable that the liposomes have both good entrapment efficiency and good drug retention *in vivo*. The lipid composition factors which effect drug retention and entrapment are discussed above. In addition, the acyl chain composition of the lipids may produce toxicity effects which are unrelated to the amount of drug present in the liposomes. Experiment conducted in support of the present invention to assess the toxicity of empty liposomes (not containing entrapped drug) on laboratory animals gave the following results: (1) Liposomes composed predominantly of saturated lipids, such as DPPG and DPPC, were more toxic than liposomes formed from corresponding unsaturated phospholipids. (2) Although unsaturated lipids are less toxic, they are also much more susceptible to lipid peroxidation damage on storage, and liposomes with substantial lipid oxidation damage are considerably more toxic, in terms of LD₅₀ values, than corresponding fresh liposomes. Also, it would be expected that lipid oxidation damage would increase toxicity in a drug/liposome composition, through decreased drug retention. As will be seen, an important aspect of the invention involves reducing peroxidative damage to lipid and drug components of an

anthraquinone/liposome composition by a combination of protective agents, and therefore the susceptibility of unsaturated lipids to oxidative damage is less of a concern. That is, the composition of the invention contributes to reduced toxicity, in part, by allowing the use of unsaturated lipids, which are both less toxic and show greater drug entrapment, without concomitant lipid peroxidative damage, which would increase toxicity effects.

Biodistribution and liposome targeting may be affected by liposome size, surface charge, and the presence of specific surface-bound molecules which act to target the liposomes to specific sites in the body. Of particular interest to the success of the present invention is the enhanced accumulation of drug/liposomes in certain target organs, such as liver and spleen, and the reduced accumulation in non-target organs, such as kidney and heart, where drug toxicity is largely localized. In accordance with one aspect of the invention, optical biodistribution is achieved within a selected size range of liposomes, as discussed below with reference to liposome sizing.

15 C. Protective Agents

The interaction of an anthraquinone drug with ferric iron in the presence of lipid, and the chemical modification of the drug which can occur in vitro have been described in Section A. As noted there, the drug modification reaction occurred under anoxic conditions, and in the presence of saturated lipids only. In Section B it was seen that, for a number of reasons, natural phospholipids (containing both saturated and unsaturated phospholipids) are generally advantageous in the drug/lipid composition of the invention. Further it is reasonable to expect that the preparation and handling of the composition will involve some exposure to molecular oxygen, so that oxidative and free radical reactions which involve unsaturated lipids and oxygen can also be expected to produce lipid and drug modifications in the composition. The present section examines the oxidative and free radical mechanisms which can lead to such drug and lipid modification and demonstrates how such modification reactions can be controlled to a great extent by including in the composition a combination of lipophilic and water-soluble protective agent which act at different points in peroxidation/free radical pathways.

A scheme showing likely oxidative and free radical reactions in a composition containing an anthraquinone drug, lipid, and ferric iron is given in Figure 3. Here, as in Section A, the liposome/anthraquinone formulation is indicated by Lip/AnQ; the acyl-chain lipid components forming the liposomes may be saturated, unsaturated, or a mixture of both. The upper left portion of the figure shows formation of the Lip/AnQ-Fe(III) complex, described in Section A. In the presence or absence of oxygen and either saturated or unsaturated lipid, the events leading to drug modification are likely to involve, first, possible formation of a semi-quinone-Fe(II) complex, and inter-or intramolecular free radical damage to the drug. Since the modified drug (DXR) is considerably more hydrophobic than the original molecule, it is possible that modification involves cleavage of the hydrophilic aminoglycoside residue from the anthracene ring. The reaction may also involve electron transfer from the semi-quinone to other functionalities in the ring structure, particularly to the hydroquinone groups, acting to delocalize the radical on the anthracene ring. The conversion of the AnQ-Fe(III) complex to modified drug is shown at the left in the figure.

Also as shown in the figure, the AnQ-Fe(II) complex can combine with molecular oxygen, leading to hydrogen peroxide formation, and ultimately hydroxyl radicals, which can then propagate free radical reactions. This oxygen-mediated pathway is suggested by earlier studies on the possible mechanisms of DXR-stimulated membrane damage in vivo where initial semi-quinone formation may occur by enzyme-catalyzed electron transfer (Goormaghtigh 1984), and in vitro, where direct oxygen participation was involved (Gutteridge 1984a).

Where the liposomes contain an unsaturated lipid (UL), a separate pathway involving oxidation and free radical damage may also be involved, as illustrated at the right in Figure 3. Initial reaction of the lipid with molecular oxygen leads to an oxygenated species which can complex with ferric iron, as indicated. The lipid complex, after undergoing a redox reaction to form an iron/lipid-radical complex, can form both peroxide and oxide lipid radicals, which in turn can propagate free radical reactions in the lipid and drug components of the composition, leading to drug and lipid modifications, as indicated. This pathway involving lipid oxidation and free radical propagation is suggested by studies on the mechanism of peroxidative damage in liposomal membranes (see, for example, Gutteridge 1984b and Sunamoto).

Heretofore, attempts to limit peroxidative damage in liposomes have focused on free radical scavenging, typically by including in the liposomes a lipophilic free radical scavenger, such as α -T. In theory, such free radical scavengers have the capacity to block free radical propagation in lipids and lipid-associated drugs, such as anthraquinones, and therefore to limit peroxidation-relative damage to that produced by

"early" radical formation reactions. Assuming that the radical-forming events are relatively benign in comparison to free radical propagation, it would be expected that little additional protection, above that provided by α -T, could be obtained. Therefore an important aspect of the present invention is the discovery that significantly greater reduction in lipid and drug modification in an anthraquinone/liposome composition can be achieved by a combination of lipophilic free radical quencher and a water-soluble protective agent which acts at the level of free radical formation.

The lipophilic free radical scavenger used in the composition of the invention is preferably α -T, or a pharmacologically acceptable analog or ester thereof, such as α -T succinate. Other suitable free radical scavengers include butylated hydroxytoluene (BHT), propyl gallate (Augustin), and their pharmacologically acceptable salts and analogs. Additional lipophilic free radical quenchers which are acceptable for parenteral administration in humans, at an effective level in liposomes, may also be used. The free radical quencher is typically included in the lipid components used in preparing the liposomes, according to conventional procedures. Preferred concentrations of the protective compound are between about 0.2 and 2 mole percent of the total lipid components making up the liposomes; however, higher levels of the compound, particularly α -T or its succinate analog, are compatible with liposome stability and are pharmacologically acceptable.

The water soluble protective is an iron-specific chelating agent selected from the class of natural and synthetic trihydroxamic acids and characterized by a very high binding constant for ferric iron (on the order of 10^{30}) and a relatively low binding constant for 2-valence cations, such as calcium and magnesium. A variety of trihydroxamic acids of natural origin have been described, including compounds in the ferrichrome class, such as ferrichrome, ferrichrome A, and albomycin; compounds in the ferrioxamine class, including the ferrioxamines and ferrimycines; and compounds in the fusaramine class. The structure and iron coordination properties of these compounds have been reviewed (Emery).

One preferred chelator is ferrioxamine B, also known variously as ferrioxamine, deferoxamine, desferrioxamine B, and Desferal. This compound shows exceptional iron binding affinity and has been proven safe for parenteral use in humans in treating iron-storage diseases and iron-poisoning (Keberle). The structure of iron-coordinated deferoxamine is shown in Figure 4. As seen, the compound has three hydroxamic acid groups ($-C(O)N(O)-$) which can arrange octahedrally about the chelated iron, to form six symmetrical Fe-oxygen ligand bonds. The binding constants of ferrioxamine for iron and several 2-valence metals, including Fe^{2+} , are given in Table V.

TABLE V

	Metallic Ion	Binding Affinity
	Fe^{3+}	10^{31}
	Ca^{2+}	10^2
	Mg^{2+}	10^4
	Sr^{2+}	10
	Zn^{2+}	10^{11}
	Ni^{2+}	10^{10}
	Co^{2+}	10^{11}
	Cu^{2+}	10^{14}
	Fe^{2+}	10^{10}

The chelating agent is present in the composition at a concentration which is in molar excess of the ferric iron in the liposome suspension. Typically, aqueous media used in liposome preparation contains at least about 1-2 μ M ferric iron, and may contain up to 100 μ M or more ferric iron. For aqueous medium containing up to about 20 μ M iron, concentrations of chelating agent of about 50 μ M are preferred.

The chelating agent is preferably added to vesicle-forming lipids at the time of liposome formation, so that the lipids are protected against drug-promoted lipid oxidation damage during liposome preparation.

Methods for preparing liposomes by addition of an aqueous solution of chelating agent are described below. Here it is noted only that the liposome suspension formed by this method contains chelating agent both in the bulk aqueous phase and in the encapsulated form, i.e., within the aqueous internal liposome region. Alternatively the chelating agent may be included in the suspension after liposome formation.

Experiments conducted in support of the present invention have examined the degree of protection of lipid and drug components in a composition containing both α -T and ferrioxamine. In one study, liposomes containing DXR and α -T were prepared substantially as in Example 1, either in the presence or absence of 50 μ M ferrioxamine. The drug/liposome composition was then stored under anoxic conditions for 1 day at 4°C, and the lipids were extracted conventionally by chloroform/methanol. The extracted lipids were dissolved in heptane and the UV spectra (190-300 nm) were monitored with a double-beam spectrophotometer. Figure 5 shows the spectra obtained for lipids incubated in the presence (spectrum A) or absence (spectrum B) of chelator. The Figure 5B spectrum shows strong peaks at about 233 nm, corresponding to conjugated diene formation and at about 275, corresponding to conjugated triene formation. The solid line spectrum in Figure 5A shows little or no evidence of diene or triene formation, and spectral subtraction shows that diene and triene formation was inhibited more than about 99% in the ferrioxamine-containing composition.

In a second study, DXR/liposomes were prepared substantially as described in Example 1, under nitrogen atmosphere or air, and in the presence or absence of α -T and ferrioxamine, as indicated in Table VI below. As in the experiment described above, the liposomes were composed of PG:PC:cholesterol, in a mole ratio of 3:7:4. α -T, when included, was present at a concentration of about 1.5 mole percent, and ferrioxamine, when included, was present at a concentration of 50 μ M. The liposomes were stored under anoxic conditions for 1 day at 4°C, as above. Chemical modification of the drug was detected both by fluorescence emission spectroscopy and by assaying the change in drug toward a more lipophilic species, as described in Section A above.

TABLE VI

	Composition						Hydrophilic	Fluorescence
	PG	PC	CHOL	α T	Desferal	Air	DXR	Intensity Ratio
	(mole ratio) (50 μ M)						%	(590/560 nm)
35	3	7	4	-	-	-	67 \pm 2	1.4 \pm 0.1
	3	7	4	+	-	-	68.1 \pm 2	1.5 \pm 0.1
	3	7	4	-	+	-	67.1 \pm 2	1.6 \pm 0.1
40	3	7	4	+	+	-	86 \pm 2	1.9 \pm 0.1
	3	7	4	-	-	+	47 \pm 3	1.3 \pm 0.1

The righthand column in Table VI shows the 590/560 nm fluorescence intensity ratios of the hydrophobic derivative of DXR for the five compositions. The data indicate two types of drug modifications, as discussed above: (1) possible cleavage of the sugar moiety to give a more hydrophobic DXR product, and (2) damage to the anthracene ring which affects the 590/560 ratio of the drug. As will be recalled from section A, the 590/560 ratio is largest in unmodified DXR, and decreases with anthracene ring modification. The ratios observed show that α -T alone and ferrioxamine alone both gave moderate protections against drug modification, with ferrioxamine having a greater protective effect. Significantly, the combination of α -T and ferrioxamine provided much greater protection than either protective agent alone, or what would be predicted from a sum of the two individual agent effects. The 1.9 value observed was substantially the same as that observed immediately after liposome preparation under nitrogen, indicating nearly complete protection of the drug through the storage period.

The studies just reported suggest that the protective mechanism of ferrioxamine is qualitatively different than that of α -T. It is likely that ferrioxamine acts primarily to inhibit peroxide generation and other "early" free-radical generating events, whereas α -T acts to quench free-radical reactions being propagated within the lipid bilayer. The likely points of inhibition by trihydroxamic acid chelator are indicated by double lines,

in Figure 3.

The inhibitory effect of trihydroxamic acid chelation on peroxidation damage in liposomes is also in contrast to the peroxidation-stimulating effect observed when a conventional tetraacetic acid-type chelator, such as ethylenediaminetetraacetic acid (EDTA), is added to liposomes. The observed difference between trihydroxamic acid and tetraacetic acid chelators, in their ability to protect against peroxidative damage in liposomes, may be due to the difference in number of iron coordination sites available in the two species. Whereas a trihydroxamic chelator is able to form octahedral ligand bonding to all six ferric iron coordination sites, tetraacetic acid chelators are limited to four-site binding, leaving two iron coordination sites available for participation in redox reactions. In this regard, it is noted that DTPA, a pentaacetic acid chelator, also shows a protective effect on anthraquinone modification *in vitro*, in the presence of lipids and ferric iron, suggesting that five-site coordination may also be effective in reducing iron participation in redox reactions.

D. Liposome Preparation

Methods for preparing drug-containing liposome suspensions generally follow conventional liposome preparation methods, such as those reviewed by Szoka et al. In one preferred method, vesicle-forming lipids are taken up in a suitable organic solvent or solvent system, and dried *in vacuo* or under an inert gas to a lipid film. The drug is preferably included in the lipids forming the film. The concentration of drug in the lipid solution is preferably in molar excess of the final maximum concentration of drug in the liposomes, to yield maximum drug entrapment in the liposomes. In preparing the liposome/DXR composition described in Example I, a film of lipids was hydrated with an aqueous suspension of DXR at a final drug-to-phospholipid concentration of about 2:10. This produced vesicles having a DXR concentration, after removal of free DXR, of about 10 mole percent.

The aqueous medium used in hydrating the dried lipid or lipid/drug is a physiologically compatible medium, and preferably pyrogen free physiological saline, such as is used for parenteral fluid replacement. The solution is mixed with a sterile solution of chelating agent, to a final desired chelator concentration, added to the film, and the lipids allowed to hydrate under rapid (with shaking) or slow (without shaking) conditions. The lipids hydrate to form a suspension of multilamellar vesicles (MLVs) whose sizes range typically between about 0.5 microns to 10 microns or greater. In general, the size distribution of MLVs in the above procedure can be shifted toward smaller sizes by hydrating the lipid film more rapidly, with shaking. The liposomes contain encapsulated chelating agent, at a concentration approximately equal to the bulk aqueous phase concentration.

E. Liposome Sizing

The liposome suspension may be sized to achieve a selective size distribution of vesicles in a size range less than about 1 micron and preferably between about 0.05 to 0.5 microns, and most preferably between about 0.05 and 0.25 microns. The sizing serves to eliminate larger liposomes and to produce a defined size range having optimal pharmacokinetic properties.

Several techniques are available for reducing the sizes and size heterogeneity of liposomes. Sonicating a liposome suspension either by bath or probe sonication produces a progressive size reduction down to small unilamellar vesicles (SUVs) less than about 0.05 microns in size. A sonicating procedure used in reducing liposome sizes to about 0.2 microns or less is described in Example I. Homogenization is another method which relies on shearing energy to fragment large liposomes into smaller ones. In a typical homogenization procedure, MLVs are recirculated through a standard emulsion homogenizer until selected liposome sizes, typically between about 0.1 and 0.5 microns, are observed. In both methods, the particle size distribution can be monitored by conventional laser-beam particle size discrimination.

Extrusion of liposomes through a small-pore polycarbonate membrane is an effective method for reducing liposome sizes down to a relatively well-defined size distribution whose average in the range between about 0.1 and 1 micron, depending on the pore size of the membrane. Typically, the suspension is cycled through the membrane several times until the desired liposome size distribution is achieved. The liposomes may be extruded through successively smaller-pore membranes, to achieve a gradual reduction in liposome size.

Centrifugation and molecular sieve chromatography are other methods which are available for producing a liposome suspension with particle sizes below a selected threshold less than 1 micron. These two methods both involve preferential removal of larger liposomes, rather than conversion of large particles to

smaller ones. Liposome yields are correspondingly reduced.

Studies aimed at determining the biodistribution and drug clearance of DXR/liposomes as a function of liposome size have been conducted, and reported earlier by the inventors (Gabizon 1982, 1983). More recent studies conducted in support of the application looked at organ distribution of DXR 1, 5, and 24 hours after administration of DXR either in free form or in liposomes having median sizes of 35 ± 14 nm or 115 ± 25 nm. The results of the study are given in Table VII, where data on each time point represents an average of three animals. The organ weights, given in parentheses, are also calculated as an average of three animals.

Table VII

	Organ	DXR Delivery System	TIME (μ g DXR/g tissue)		
			1 hr	5 hrs	24 hrs
15	Liver (1.46g)	115nm vesicles	44.9	37.1	16.7
		35nm vesicles	39.2	16.8	4.0
		Free	30.5	16.4	3.8
20	Spleen (0.15g)	115nm vesicles	24.6	26.0	16.0
		35nm vesicles	14.6	13.5	8.0
		Free	8.1	10.7	8.8
25	Heart (0.12g)	115nm vesicles	2.3	2.0	1.1
		35nm vesicles	5.9	3.4	1.2
		Free	8.4	4.2	0.9
30	Lungs (0.16g)	115nm vesicles	4.3	3.8	2.3
		35nm vesicles	8.9	6.9	3.1
		Free	13.2	8.6	3.6
	Intestine	115nm vesicles	2.9	1.0	1.3
		35nm vesicles	6.3	5.6	2.8
		Free	5.8	3.1	1.9
35	Kidney (0.22g)	115nm vesicles	12.1	6.0	3.8
		35nm vesicles	26.4	13.6	6.0
		Free	31.9	19.4	6.9
40	Skeletal Muscle (-)	115nm vesicles	0.47	3.7	0.36
		35nm vesicles	1.6	1.1	0.69
		Free	2.3	2.8	0.59

The data in Table VII show that liposomes, and particularly the 115 nm liposomes, show increased drug levels in the liver and spleen and decreased drug levels in heart, lung, intestine, kidney, and skeletal muscles when compared with the free drug. The liposomes are thus particularly advantageous in treating liver- and spleen-localized tumors and in reducing toxicity related to drug levels in non-target tissues, particularly the heart. Another important advantage of 115 nm liposomes which is seen from the data is slower drug clearance in liver and spleen tissue. The drug clearance in animals which received the smaller (35 nm) liposomes more closely followed that of the free drug. The improved biodistribution and longer clearance times observed with the 115 nm vesicles are observed in the liposome size range down to about 0.05 microns. Below this range, drug distribution and clearance characteristics begin to approximate those of the free drug, as seen. It has also been found that the drug loading factor (amount of drug/liposome lipid) is relatively low in SUVs, presumably due to the strained configuration of SUV bilayers, and that SUVs are less stable in plasma than larger-size liposomes.

For a variety of reasons, the optimal upper size limit of the liposomes is about 0.5 microns and, preferably, about 0.25 microns. First, the desired target tissue regions, such as liver sinusoids and parenchyma, spleen, and bone marrow are more accessible to liposomes smaller than about 0.25 microns.

Secondly, liposomes in the 0.25 micron size range can be readily sterilized by filtration through a depth filter. Smaller vesicles also show less tendency to aggregate on storage, thus reducing a potentially serious problem when the composition is administered parenterally. Finally, liposomes which have been sized down to the submicron range show a more uniform biodistribution and drug clearance characteristics, since they have more uniform sizes.

F. Removing Free Drug

Free drug, i.e., drug present in the bulk aqueous phase of the medium, is preferably removed to increase the ratio of liposome-entrapped to free drug. This is particularly useful if the liposomes have been prepared under conditions of excess drug, to maximize drug/lipid ratios in the liposomes. Under the preparation conditions described in Example I, for example, the liposomes incorporated between about 50-60% of the total DXR added initially, leaving 40-50% free DXR. The drug removal is designed to reduce the final concentration of free to less than about 20% and preferably, less than about 10% of the total drug present in the composition.

Several methods are available for removing free anthraquinone drug from a liposome suspension. As indicated above, the sized liposome suspension can be pelleted by high-speed centrifugation, leaving free drug and very small liposomes in the supernatant. Alternatively, gel filtration can be used to separate larger liposome particles from solute (free drug) molecules. Of course, it is necessary to include the chelating agent in the elution buffer, or add chelating agent to the eluted liposomes, since the gel matrix itself will remove the chelation agent from the original suspension.

One preferred procedure for removing free drug utilizes an ion-exchange resin capable of binding drug in free, but not in liposome-bound, form. With DXR, the preferred resin is a cation-exchanger, since the drug is positively charged at neutral pH. One advantage of the ion-exchange approach is that free drug can be removed in line during drug administration, as the drug composition flows from a container to the site of injection. This system has the advantage that essentially all of the free drug can be removed from the suspension, and the liposome administered before drug leakage from the liposome to the aqueous bulk phase occurs. The system may also be effective in removing free pyrogens, with proper selection of resin(s).

II. Therapeutic Uses in Tumor Treatment

Studies by the inventors, discussed above, have shown that small, drug-carrying liposomes can concentrate liposome-associated drug in tissue regions, such as liver sinusoids and parenchyma, spleen, and bone marrow which are accessible to small liposomes (less than about 0.2 microns in size). These findings having important implications for treating metastatic diseases of the liver, primary hepatomas, lymphoid proliferative diseases, and leukemias, all of which are major cancers, both in terms of numbers and geographic distribution.

One of the most effective anti-tumor drugs, and a drug of choice in many instances, is DXR, a related anthracycline glycoside compound, or mitoxanthrone. For these anthraquinone drugs, the challenge for improved anti-tumor therapy is to decrease the toxicity and side effects of the liposome therapeutic composition without compromising the therapeutic effect of the drug. With regard to DXR, which has a very specific toxicity for the heart muscle, the inventors have shown that a DXR/liposome composition, properly formulated, can significantly decrease the uptake of drug by the heart muscle and reduce the severity of the histopathological changes seen in mice treated with similar doses of free DXR.

Another source of toxicity is oxidative damage to the lipid and drug components of the liposome composition. As demonstrated in Section I, anthraquinone drugs actively promote peroxidative reactions in the presence of lipids, leading to significant modification of both lipid and drug components in the drug/liposome composition. The modified composition is more toxic, due to the peroxidative damage to lipids and increased drug leakage, and the drug itself may lose therapeutic effectiveness with modification.

The toxicity and therapeutic effectiveness of a DXR/liposome composition (L-DXR) prepared according to the invention is examined in Examples II-V. Example II show that significantly higher doses of DXR in liposomal form can be administered without lethality. The results are summarized in Figure 7. Example III, which looks at the effects of repeated administration of graded doses of free drug (F-DXR) and L-DXR finds significant reduction in (a) lethality, (b) loss of body weight, (c) changes in blood cholesterol and triglyceride levels, and (d) organ pathology when L-DXR treatment is compared with F-DXR.

Clinical trials using L-DXR to treat patients suffering from various types of cancer including metastatic and primary liver cancer, have been carried out. Initial clinical trials are reported in Example IV. The findings there indicate a significant improvement in overall patient comfort, due to significant reduction in the incidence of gastrointestinal toxicity (nausea, vomiting, diarrhea, and stomatitis).

5 Extended clinical trials, to investigate drug toxicity and side effects with therapeutically effective doses of L-DXR are reported in Example V. These studies show significantly reduced incidence nausea and vomiting, from free-drug levels of between about 20-55% to about 6%, and a significant reduction in the incidence of total and partial alopecia, from reported free-drug level of between about 85-100%, to about 46%. The doses of L-DXR which were administered in the trials were comparable to free-drug doses used
10 in cancer chemotherapy in humans. The invention thus provides, in one aspect, a method of significantly reducing the incidence of nausea and alopecia which are associated with intravenous administration of doxorubicin to humans, in bolus-injection form. The method includes entrapping doxorubicin in liposomes, and administering the liposomes intravenously, by fast-drip injection, in a therapeutically effective quantity. As defined herein, the term "fast-drip" is intended to include intravenous or intra-arterial administration in
15 bolus form or in drip form that requires between several minutes up to a few hours for introduction of a therapeutically effective dose of the L-DXR formulation.

Pharmacokinetic studies conducted in support of the invention suggest that liposomal delivery of DXR follows a slow release pattern. Typical release kinetics of DXR administered in free (solid lines) and liposomal (dotted lines) form is shown in Figure 9. The data were obtained by analysing plasma samples
20 from patients in the extended clinical trials (Example 5). The drug concentration curves shown in the figure were analysed by best-fit to a bi-exponential curve, described by a rapid distribution phase, and a slower terminal phase. As seen, both free and liposomal DXR have about the same half life through the distribution phase, calculated to be about 10-15 minutes. In the terminal phase, however, the half life of the free drug is about 25 hours, whereas that for liposomal drug is about 67 hours (the mean of six studies). From this data,
25 it can be appreciated that the liposomal formulation is effective in producing a controlled slow release of drug into the bloodstream.

The clinical trials reported in Example V also show that the method of the invention for administering DXR in liposomal form is therapeutically effective against both primary and secondary neoplasms of the liver. Therapeutic responses in about one-half of the patients with primary or secondary neoplasms of the
30 liver were observed, both where the L-DXR was administered intravenously or intra-arterially. All of the patients in the trials were or had become refractory to other treatment modalities, including in some cases, administration of free DXR intravenously or intra-arterially. Thus the invention includes, in another aspect, a method of treating primary and secondary neoplasms of the liver by the steps of: (a) providing an aqueous suspension of liposomes whose sizes are predominantly between about 0.05 and 0.25 microns, and which
35 contain at least about 2.5 mole percent DXR or a pharmacologically acceptable analog, derivative, and (b) administering a pharmacologically acceptable amount of the composition to the patient via the bloodstream.

The following examples illustrate various methods and compositions of the invention, but are in no way intended to limit the scope thereof.

40

Materials

Adriamycin (doxorubicin HCl, containing lactose) was obtained from Farmitalia (Milano, Italy); phospholipids (chromatographically tested, greater than 99% purity from Lipid Products (Surrey, England)
45 or Avanti Lipids (Birmingham, AL); cholesterol (chromatographically tested, greater than 99% purity); physiologic saline solution, from Baxter Travenol (Ashdod, Israel); ferrioxamine (Desferal), from Ciba-Geigy (Basel, Switzerland); and (α -T), from Sigma Chemicals (St. Louis, Missouri).

50 Example I

Preparation of Liposome-Entrapped DXR (L-DXR)

A solution of vesicle-forming lipids contained 360 mg cholesterol, 33 mg α -T, 1750 mg egg PC, and
55 750 mg PG (egg, sodium salt) in 100 ml chloroform. The mole ratio of lipid components was PC:PG:cholesterol (7:3:4). The solution was divided, added to two 500 ml round-bottom flasks, and evaporated under vacuum in a rotary evaporator. Further removal of organic solvents and drying of the film was accomplished by lyophilization in a lyophilizer at 150 mTorr pressure overnight.

A drug solution contained 300 mg DXR (6 vials containing 50 mg doxorubicin HCl and 250 mg lactose each) dissolved in saline (sterile, pyrogen-free physiologic NaCl solution) containing ferrioxamine (~50 μ M). The final solution contained DXR at 5 mg/ml, and ferrioxamine at 30 μ g/ml in a total volume of 60 ml. The DXR solution was added to round-bottom flasks containing the lipid films (30 ml/flask). This initial phospholipid-to-DXR molar ratio was 10:2. The mixture was vortexed, then equilibrated in a shaking device at 100 stroke/min for 2 hr at 15-20°C.

The multilamellar vesicle (MLV) dispersion obtained was sonicated by ultrasonic irradiation using a 1/2-in sapphire-bonded probe, with pulse sonication during 15 min (60%, or 9 minute, sonication time) under optimal output conditions. Sonication was carried out under a stream of nitrogen (or argon) with the vessel immersed in ice water. The sonication procedure was standardized by determining the optical density of the dispersion at 380 nm wavelength. Specifically, the sonication was carried out until a 1:100 dilution of the dispersion in saline gave an O.D. value of less than 0.9. If the O.D. was greater than 0.9, the sonication was continued for an additional 3 min. Starting O.D. values of the MLV dispersions are in the range of 1.300-1.500. The sonicated dispersions were centrifuged in a bench-top centrifuge (2,000 rpm, 10 min) to remove large titanium particles. Alternatively, the liposomes were extruded several times through 0.2 μ m pore polycarbonate membrane until an O.D. value of less than 0.9 was obtained. Free drug was removed by ultracentrifugation. The sized liposomes were sterilized by filtration through a 0.2 micron polycarbonate filter.

The sterilized liposomes were stored in vacuum-sealed sterile glass bottles at 4°C in the dark. The DXR-containing liposomes (L-DXR) had the following characteristics:

- (a) Total DXR in the liposomes was greater than 50% of the initial amount of drug;
- (b) Phospholipid concentration (determined by organic phosphate present) was 10-13 μ mole/ml;
- (c) The amount of free DXR (determined by DXR/phospholipid molar ratio before and after passage through Sephadex G-50 column) was less than 5% of the total DXR;
- (d) The size distribution was between 0.05 and 0.2 microns;
- (e) Lipid oxidation (determined by a modification of the method of Klein) was less than 1% of total phospholipid, based on the abnormal spectral absorbance at 233 nm, due to diene conjugation;
- (f) Stability in human plasma (measured by DXR retention in DXR liposomes in 50% plasma at 37°C for 1 hour) was greater than 80%;
- (g) The liposomes were sterile, as determined by addition (3-4 ml) of the liposome suspension in Bactec 7-ml bottles (aerobic and anaerobic microbiological cultures) for 7 days; and
- (h) The rabbit pyrogenicity test was negative.

The liposome suspensions were stable for three months at refrigerator temperature, without significant changes in any of the above characteristics.

Example II

Single Injection Survival with Graded Doses of F-DXR and L-DXR

L-DXR was prepared as in Example I, to final DXR concentrations of 1, 1.5, 2, and 2.5 mg/ml, with corresponding phospholipid concentrations of 16, 24, 32, and 40 μ mole/ml, respectively, and cholesterol concentrations of 6.4, 9.6, 12.8, and 16 μ mole/ml, respectively. Free DXR (F-DXR) was prepared in sterile, pyrogen-free physiologic saline solution to final concentrations of 1, 1.5, 2, and 2.5 mg/ml.

BALB/c male mice were treated intravenously with 4 different doses of F-DXR and L-DXR: 10, 15, 20, and 25 mg/kg, by administering about 1% total animal weight of the corresponding F-DXR or L-DXR formulation. The survival curve recorded for a period of 90 days is shown in Figure 7. As seen, there is a marked decrease in toxicity of the drug in the liposome-encapsulated form. No mortality was registered among animals treated with 15 and 10 mg/kg of L-DXR and 60% of the mice injected with 20 mg/kg survived throughout 90 days. F-DXR caused 100% of mortality in the groups injected with 25, 20, and 15 mg/kg and 20% mortality in the group receiving 10 mg/kg.

The pattern of mortality was a mixed one for some mice, especially those from the high-dose treated groups, dying within the first two weeks after injection (one month in the case of the 25 mg/kg L-DXR treated group), and other mice dying with a significant delay of 6-7 weeks or more after drug administration. Autopsy of mice dying in the acute phase showed severe gastrointestinal toxicity with hypersecretion and dilation of the intestinal loops, while in those mice dying in the delayed phase, the most significant findings were decrease of body weight and hypotrophy of the abdominal organs.

Example IIIEffects of Repeated Administration of Graded Doses of F-DXR and L-DXR

5 L-DXR was prepared as in Example I, to final DXR concentrations of 0.5, 0.75, and 1.0 mg/ml, corresponding to phospholipid concentrations of 8, 12, and 16 μ mole/ml, respectively, and cholesterol concentrations of 3.2, 4.8, and 6.4 μ mole/ml, respectively. Free DXR (F-DXR) was prepared in sterile, pyrogen-free physiologic saline solution to final concentrations of 0.5, 0.75, and 1 mg/ml.

10 Sabra mice were divided into groups of 15 mice. Three different doses of either F-DXR or L-DXR were tested: 5, 7.5, and 10 mg/kg body weight. Each dose level was tested on 15 male and 15 female mice. Treatment was administered as a bolus injection through the tail vein on days 0, 14, 28, 42, 56, 70, 84, and 98 of the experiment. The pulsed injection scheme was used for the purpose of achieving high cumulative doses, prolonging the period of exposure of the animals to the drug, and mimicking the way DXR is usually administered in clinical practice. In view of the toxicity observed after single injection (Example XI), DXR was administered at a maximum 10 mg/kg dosage so that the occurrence of acute toxicity would be reduced or nil. In order to avoid the overlapping myelosuppression of successive treatment, 14 days were allowed between successive injections, based on previous observations that full regeneration of the activity of myeloid centers in the spleen after treatment with 10 mg/kg of DXR occurred between 7-12 days after drug administration.

20 During the course of the experiment, mice were inspected daily and survival curves were recorded. Whenever possible, dead mice were autopsied and a limited sample of organs sent for histopathological examination. Blood counts and body weights were checked weekly. The blood counts were done in a Coulter analyzer with a 20 μ l sample obtained from the retro-orbital blood sinuses. One-third of the mice of each group were sacrificed on day 42, after 3 injections. A second third was sacrificed on day 84, after 6 injections. The remaining mice were sacrificed on day 180, 82 days after the 8th and last injection. Before sacrifice, the mice were bled under light ether anesthesia to obtain serum for the biochemical tests. The animals were then dissected and the removed organs, after weighing, were fixed in Bouin's solution and processed for light microscopy examination with hematoxylin-eosin-phosphomolybdic acid: light green stain.

30

(a) Effects on Survival.

The survival curves of male and female mice are shown in Figures 8 and 9. Significant mortality rates were observed in all the groups infected with free drug. By contrast, 100% survival after 180 days was found in the groups injected with the intermediate (7.5 mg/kg) and low dose (5 mg/kg) of L-DXR. The high dose (10 mg/ml) of L-DXR caused a substantial rate of mortality, although a minor group of the animal survived a cumulative dose of DXR of 80 mg/kg. There was a slight trend towards higher toxicity in male as compared to female mice with the high and intermediate dosages of F-DXR.

40

(b) Effects on Peripheral Blood Counts.

Blood counts taken 7 and 14 days after each injection showed minor reductions only in the white cell count. There were no significant differences between the F-DXR and the L-DXR treated groups. The hemoglobin concentration was not appreciably affected during the course of the experiment.

45

(c) Effects on Body and Organ Weights.

50 Table II shows the effect on body and organ weights of repeated administration of F-DXR or L-DXR, at a 10mg/kg dosage level. The weight measurements were made 42, 84, and 180 days after initial drug injection. The body and organ weights are expressed in percent relative to untreated mice. The percent survivors at each of the three points is indicated at the top in the table; the dashes (-) indicate no survivors.

When the weights of liver, spleen, kidneys, and heart of the different experimental groups are compared, it is apparent that the F-DXR caused a substantial weight reduction noticeable after 3 injections (day 42) and 6 injections (day 84). L-DXR treatment caused no significant decrease of organ weights, except in male mice on day 180.

Unlike the acute toxicity deaths observed in the animals injected with single high doses of DXR

(Example XI), the deaths observed with the multiple injection regime were generally characterized by a decrease of body weight with disappearance of abdominal and subcutaneous fat, organ shrinkage, and lack of gastrointestinal toxicity. The lower the dose received, the less pronounced were the body and organ weight reductions of dead mice. All male and female mice which received multiple doses at 7.5 or 5 mg/kg L-DXR survived to day 180. Animals dying after treatment with 7.5 and 5 mg/kg of F-DXR frequently exhibited pleural and peritoneal effusions, cardiomegaly, and lung congestion. A finding noticed only in mice treated with F-DXR, 10 mg/kg, was a marked weakness of the lower limbs unexplained by the histopathological finding in skeletal muscle and which could be related to a neuropathic effect.

Table VIII
Percent Body and Organ Weight

		DAYS AFTER 1st INJECTION					
		<u>Male</u>			<u>Female</u>		
		42	84	180	42	84	180
20	% Surviving						
	F-DXR	87	-	-	93	27	-
	L-DXR	100	90	18	100	100	40
25	Body						
	F-DXR	75	-	-	95	84	-
	L-DXR	96	94	69	97	96	83
30	Liver						
	F-DXR	75	-	-	107	91	-
	L-DXR	96	101	88	91	103	99
35	Spleen						
	F-DXR	71	-	-	78	67	-
	L-DXR	80	88	131	78	91	101

Table VIII (Continued)

		DAYS AFTER 1st INJECTION					
		<u>Male</u>			<u>Female</u>		
45	Kidneys						
	F-DXR	76	-	-	78	91	-
	L-DXR	87	81	66	93	100	84
50	Heart						
	F-DXR	82	-	-	97	74	-
	L-DXR	90	105	71	91	91	93

d) Effects on Blood Biochemistry Values

Blood levels of glucose, cholesterol, triglycerides, urea, uric acid, creatine, and total protein were measured at 42, 84, and 180 day time points. Blood enzyme levels of alkaline phosphatase, aspartate aminotransferase, and alanine aminotransferase were also assayed at these time points.

The most important differences in blood chemistry between F-DXR and L-DXR administration occurred in blood glucose, cholesterol, and triglyceride levels. These differences are seen in Table III below, which

gives data on female mice receiving 10 mg/kg of either F-DXR or L-DXR. Glucose, cholesterol, and triglyceride levels are expressed as percent control (untreated) animals, and "nm" means "not measured".

As seen, animals treated with free drug show decreased blood glucose, hypercholesterolemia, and hyperglyceridemia. By contrast, reduced blood glucose was seen only at 180 days for L-DXR treatment, and elevated cholesterol and triglyceride levels were not observed even at 180 days.

Although not shown in the table, it is also noted that animals receiving lower free drug dosages (7.5 and 5 mg/kg) showed increased creatine levels at 180 days, indicating serious renal function impairment. Increased levels of creatine were not observed in L-DXR treated mice, even at 10 mg/kg doses.

Increased liver enzymes --alkaline phosphatase and aminotransferases --were observed in animals treated with either F-DXR or L-DXR, although in both experimental groups a drop in alanine aminotransferase occurred at 180 days.

TABLE IX
Change in Blood Chemistry

		DAYS AFTER 1st INJECTION		
		42	84	180
% Surviving				
	F-DXR	93	27	1
	L-DXR	100	100	100
Glucose				
	F-DXR	97	57	-
	L-DXR	113	98	50
Cholesterol				
	F-DXR	300	383	-
	L-DXR	79	93	111
Triglyceride				
	F-DXR	nm	416	-
	L-DXR	nm	123	105

(e) Effects on Tissue Pathology.

The histopathological examinations showed reduced pathological damage, especially in kidney, heart, and ovaries in animals receiving L-DXR, compared with free drug administration.

Example IV

Clinical Trials With L-DXR

Eight cancer patients have been treated with the L-DXR composition of Example III. The administration of the drug was done using a 120 ml "Voluset" at a concentration of approximately 0.5 mg/ml in isotonic saline and at a rate of 2-3 ml per min. For intraarterial injections, peristaltic pumps were used, with the rate of infusion being ~ 1.5 ml/min. Two patients have had the liposomal drug delivered intraarterially into the liver, without any side effects. This was done taking advantage of previously implanted pumps. In both patients, the perfusion of the liver with the pump was previously examined with a Tc-99 labeled macroaggregated albumin scan.

Transient myelosuppression affecting the white blood cell count was observed in two patients: patient #3 (Table IV), after receiving the third dose (highest dose given, 100 mg DXR) and patient #6 (pretreated

with X-irradiation and with 5-fluoracil, and Mitomycin-C) after 80 mg DXR. The nadir for patient # 3 was 1,900 cells/mm³, and for patient #6, 2,300 cells/mm³.

Hair loss was significant in only 50% of the patients. Fever related to the administration of L-DXR from pyrogen-free batches was observed in two instances. The temperature rise was preceded by chills and reached 39°C to 40°C. It occurred several hours after the treatment. In both cases, it was self-remitting. With one of the two patients fever was a constant feature after administration of F-DXR. No gastrointestinal toxicity, neither immediate (nausea, vomiting, diarrhea) nor delayed (stomatitis) was observed in any of the patients. Electrocardiographic changes were not observed after treatment. No local pain in the site of injection was noticed by any patient.

In a patient suffering from hepatoma and having failed previously with several drugs, including DXR, L-DXR injected intraarterially brought about a significant shrinkage of the liver tumor. Cumulative details on the treated patients is presented in Table X below.

Example V

Extended Clinical Trials With L-DXR

Nineteen cancer patients were evaluated in additional clinical trials, to examine immediate and delayed response to single-and multiple-dose treatment with L-DXR, using the same L-DXR formulation as used in Example IV.

The criteria for patient eligibility in the study were: (a) predominant neoplastic involvement of the liver and/or the hematopoietic compartment; (b) failure of conventional therapy; (c) if the patient has previously received free DXR, the total accumulated dose of F-DXR plus L-DXR would not exceed 550 mg/mm² of body surface area; (d) no evidence of heart failure, and normal left ventricle ejection fraction, and (e) informed consent. The patient characteristics are given in Table XI below.

TABLE XI

Patient Characteristics

No. of patients treated	19
No. of patients evaluable for immediate toxicity	19
No. of patients evaluable for toxicity	15
Male/Female	11/8
Age in years -- mean (range)	53 (22-75)
Primary tumor site -- colon & rectum	6
liver (hepatocellular ca)*	5
lung (small cell ca)	2
soft Tissue Sarcoma	2
melanoma	1
stomach	1
breast	1
A.M.L.	1
Previous therapy with adriamycin, yes/no	10/9

One objective in the study was to evaluate immediate and delayed toxicity and side effects at therapeutically effective dose levels. The dose per course, and total number of courses per patient used in the trials, are given in Table XII below. As seen, dose levels of L-DXR as high as 70 mg/m² body surface area per administered. The maximum accumulated dose for a single patient was 210 mg/m². The L-DXR composition, which contained between about 0.5-1 mg DXR/ml, and between about 15-30 mg liposome phospholipid/ml, was administered intravenously, by fast drip (about 3 ml/min). Multiple courses were administered every three weeks.

TABLE XII

	<u>Dose of L-ADM per Course</u>		<u>Number of Courses per Patient</u>	
	<u>Dose mg/m²</u>	<u>Patients</u>	<u>Courses</u>	<u>Patients</u>
10	20	2	1	10
	30	3	2	5
	40	1	3	4
	50	6		
	60	4		
15	70	3		

A. Toxicity and Side Effects

The toxicity and side effects observed in the trials are summarized in Table XIII. With regard to the immediate effects, it is observed that nausea and vomiting were observed in 2 out of 32 courses (about 6%). This compares with an incidence of nausea and vomiting of between about 21-55% when free adriamycin is administered in bolus form, even at low doses. The incidence of hypotension and chills was also low, and no ECG change and pain at the site of injection was noted.

Considering the delayed side effects, it is seen that the significant alopecia occurred in 6 of the 15 patients studied. Two of the patients in this group received DXR injections of 30 mg/m² or less, and therefore are not counted in the study, since alopecia is not always observed when free DXR is given at this level. Therefore, the corrected incidence of alopecia is 6/13 or about 46 percent. This compares with the reported incidence of significant alopecia with free adriamycin dosing, which is between about 85-100%. The incidence of bone marrow depression is slightly lower than reported for free drug. The accumulated treatment dose was not at the level where chronic cardiotoxicity is observed, so comparisons with free drug are not conclusive.

TABLE XIII

<u>Toxicity and Side Effects</u>	
<u>Immediate (19 Patients -- 32 Courses)</u>	<u>No. of Cases/Courses</u>
Hypotension	1
Fever & chills	4
Nausea, vomiting	2
Chest pain, ECG changes	0
Pain in site of injection	0
<u>Delayed (15 Patients -- 28 Courses)</u>	
Alopecia	
- Partial	3
- Complete	2
Bone marrow depression -- (<3,000 WBC/ μ l)	
- Total no. observed	7
- With agranulocytosis and fever	2
Mucositis (non-agranulocytosis related)	0
- Total no. observed	1
- Hepatotoxicity	0B.

B. Therapeutic Effects

The patients with liver involvement (primary or secondary liver liver neoplasms) were additionally studied for (a) change in tumor size, as determined by imaging and (b) liver function test and tumor markers. As indicated above, the patients selected for the clinical trials had failed or ceased to respond to conventional therapy, including in two case, free DXR administration. Among the patients studied two patients with primary liver tumors and one with a soft-tissue sarcoma which had metastasized to the liver, showed showed improvement after multiple course treatment with L-DXR, as evidenced by tumor shrinkage 25-50 percent and improvement in liver function. Two of these patients had previously shown some response to free DXR, but had ceased to respond to the free drug before treatment with liposomal DXR. A fourth patient with primary liver neoplasm showed a stable response to L-DXR administration, as evidenced by a stabilization of liver function and slight shrinkage (less than 25 %) of the tumor. In total, half of the patients studied with primary or secondary liver tumors showed some improvement, even though these same patients had been refractory to other treatment modalities.

Another patient not included in the toxicity studies had been previously treated by intra-arterial injection (into the hepatic artery) with free DXR, without improvement. This patient, when treated with L-DXR administered intra-arterial showed a significance decrease in tumor size, indicating that the liposomal formulation is more effective than free drug, even when administered directly at the site.

While preferred methods and uses have been described herein, it will be appreciated that various changes and modifications can be made without departing from the invention.

Claims

1. An anthraquinone/liposome composition comprising liposomes containing at least about 0.1 mole percent of a lipophilic free-radical scavenger, entrapped in the lipid bilayer region of the liposomes, an anthraquinone drug having quinone and hydroquinone functionalities on adjacent anthracene rings, and a trihydroxamic acid chelating agent at a concentration in molar excess of ferric iron in the composition.
2. The composition of claim 1, wherein the drug is an anthracycline glycoside antibiotic.
3. The composition of claim 2, wherein the drug is doxorubicin, or a pharmaceutically acceptable analog, derivative, or salt thereof.
4. The composition of any one of the preceding claims, wherein the free-radical scavenger is alpha-tocopherol or a pharmacologically acceptable analog, derivative, or ester thereof.
5. The composition of any one of the preceding claims, wherein the trihydroxamic acid is ferrioxamine.
6. The composition of any one of the preceding claims, wherein the liposomes are in an aqueous suspension and the chelating agent is present in substantially equal concentrations inside and outside the liposomes.
7. The composition of any one of the preceding claims, wherein the liposomes are predominantly between about 0.05 and 0.2 microns in size.
8. The composition of any one of the preceding claims, wherein the liposomes contain between about 20-50 mole percent cholesterol, between about 10-50 mole percent of a negatively charged phospholipid selected from the group consisting of phosphatidyl glycerol, phosphatidylinositol and phosphatidylserine.
9. The composition of any one of the preceding claims, wherein the liposomes are in an aqueous suspension and the drug is present in the liposomes in a substantially saturating amount.
10. The composition of any one of the preceding claims, which has been treated to remove free drug not associated with the liposomes.
11. The composition of any one of the preceding claims, wherein the liposomes are in lyophilized form.
12. An aqueous suspension of liposomes whose sizes are predominantly between about 0.05 and 0.25 microns, and which contain at least about 2.5 mole percent doxorubicin or a pharmacologically acceptable analog, derivative, for use in a method of treating a primary or secondary neoplasm of the liver by administering the composition to the patient via the bloodstream.
13. The suspension of claim 12, wherein the liposomes contain at least about 0.1 mole percent of a lipophilic free-radical scavenger; and the aqueous suspension contains a trihydroxamic-acid chelating agent present in the bulk aqueous phase of the suspension, at a concentration in molar excess of ferric iron in the suspension.
14. The suspension of claim 13, wherein the chelating agent is ferrioxamine or pharmacologically acceptable analog or salt thereof.

15. The suspension of any one of claims 12 to 14, wherein the liposomes are provided to include between about 10-40 mole percent of a negatively charged phospholipid selected from the group consisting of phosphatidyl glycerol, phosphatidylinositol, and phosphatidylserine.

16. The suspension of any one of claims 12 to 15, wherein the liposomes include between about 20-50 mole percent cholesterol.

17. The suspension of any one of claims 12 to 16 which has been treated to remove doxorubicin present in unbound form in the bulk aqueous phase of the suspension.

18. Doxorubicin entrapped in liposomes for use in a method of significantly reducing the incidence of nausea and alopecia in humans produced by bolus intravenous administration of a therapeutically effective amount of free doxorubicin by administering the liposomes by fast-drip to the bloodstream.

19. Doxorubicin according to claim 18 for use in the treatment of neoplasms of the liver.

20. A liposome composition according to any one of claims 1 to 11 for use in a method of treating neoplasms of the liver.

21. A liposome or composition containing it as defined in any one of claims 12 to 19 for use in the production of a medicament for the treatment of neoplasms of the liver.

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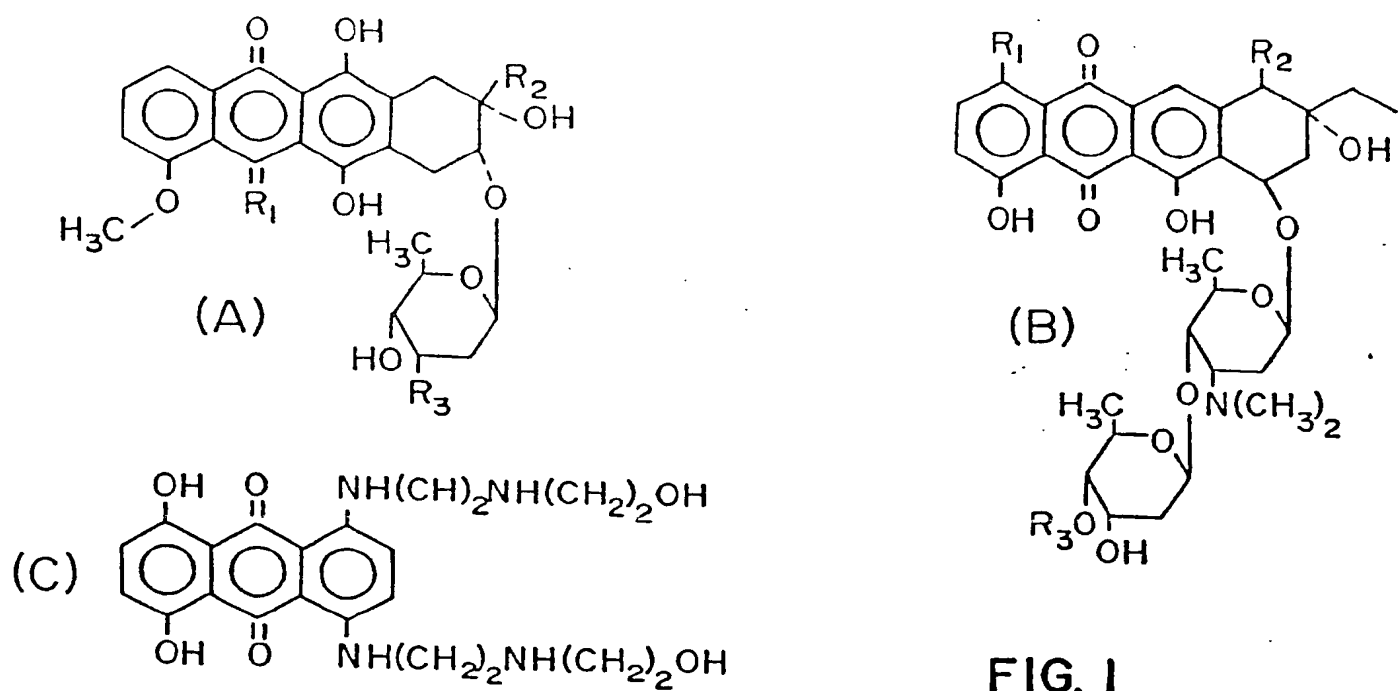


FIG. 1

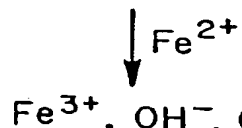
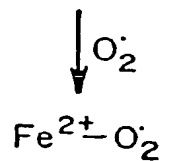
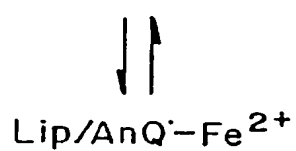
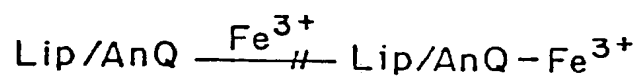


FIG. 3

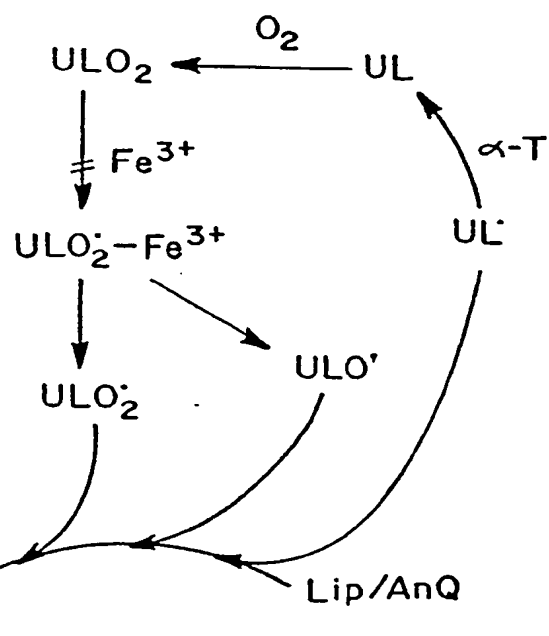


FIG. 2

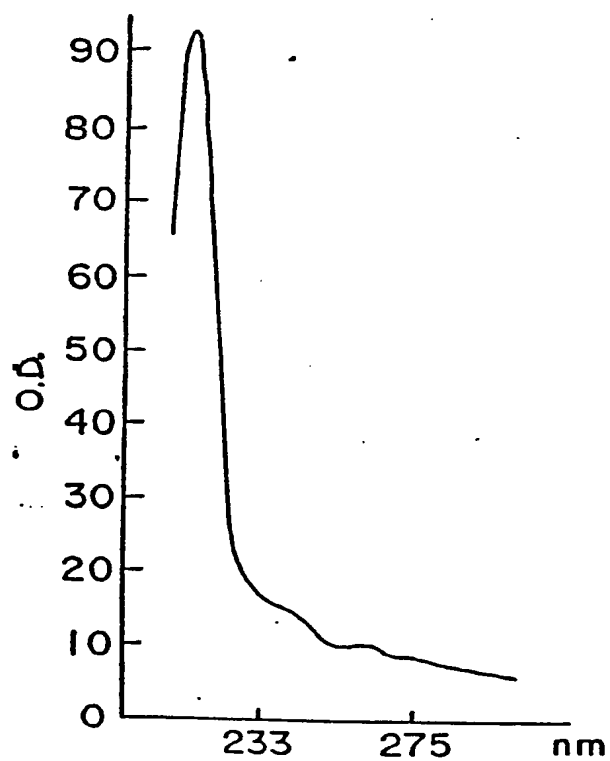
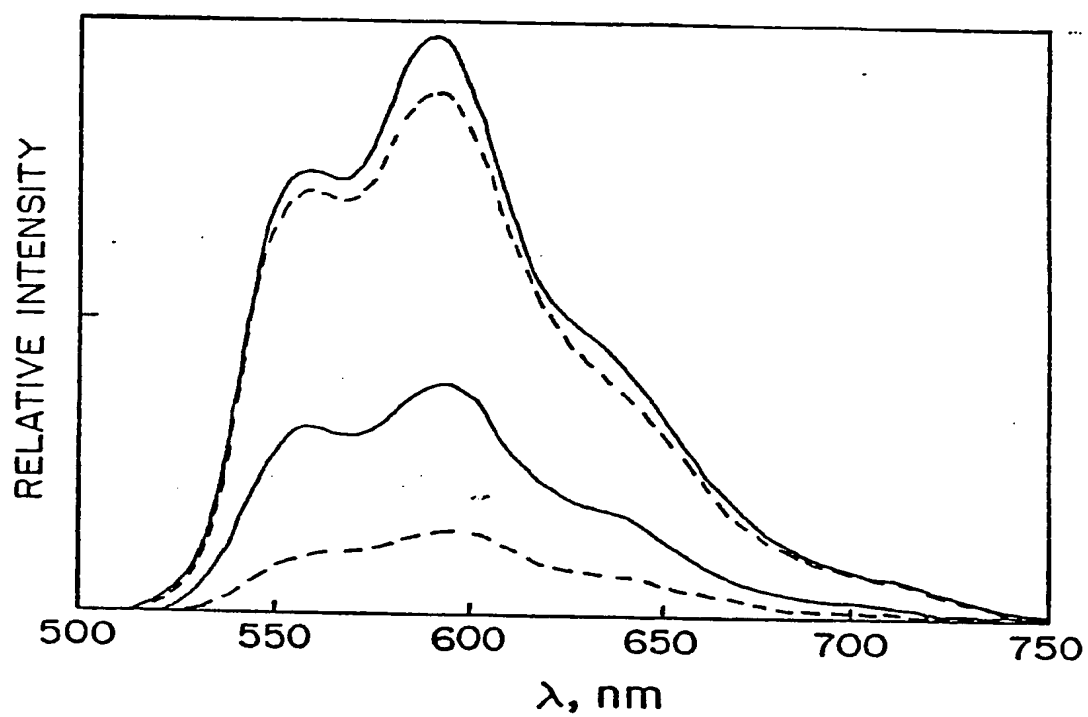


FIG. 5A

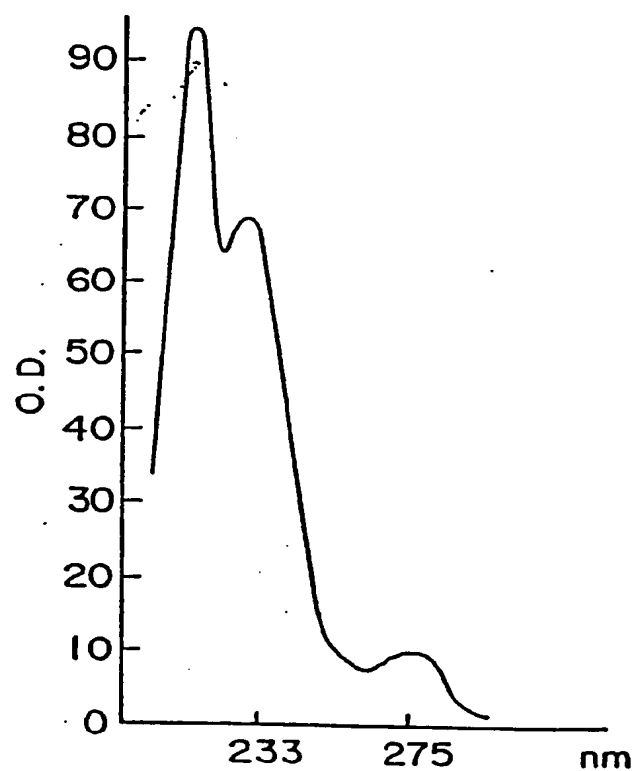


FIG. 5B

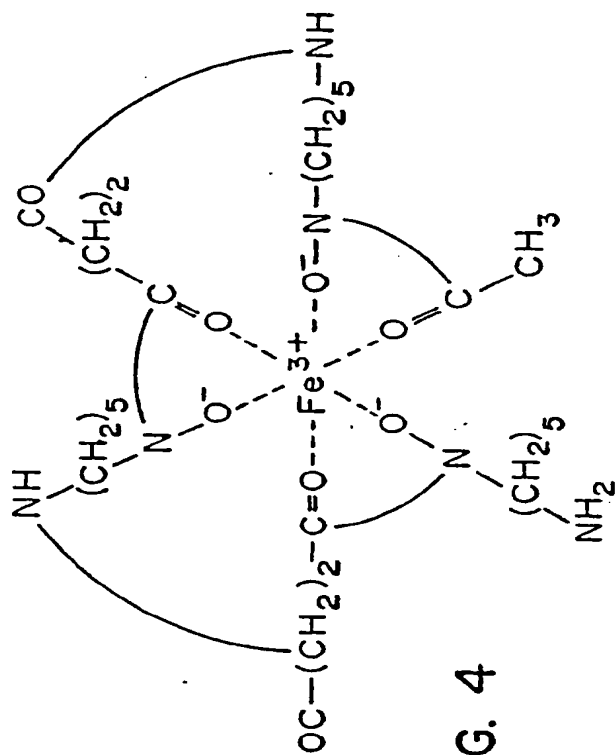


FIG. 4

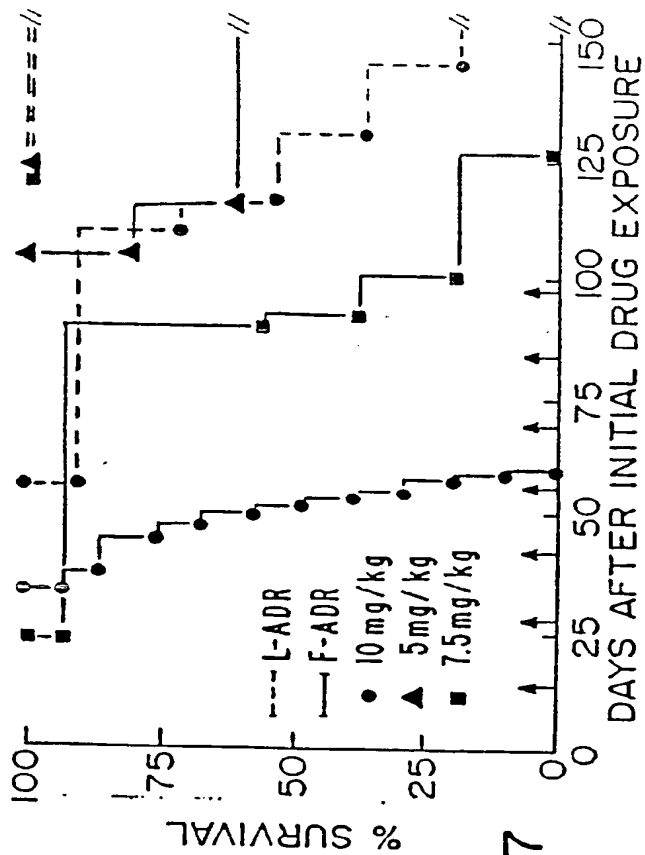


FIG. 7

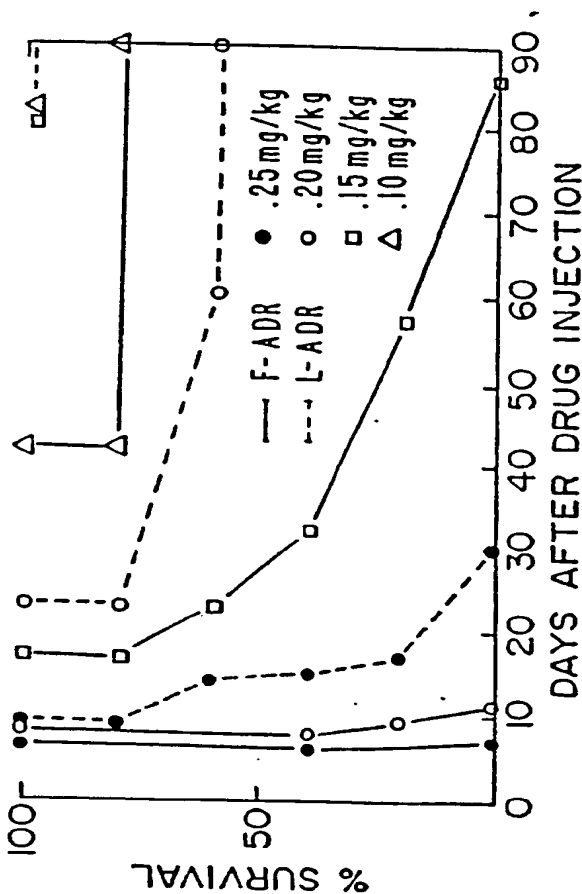


FIG. 6

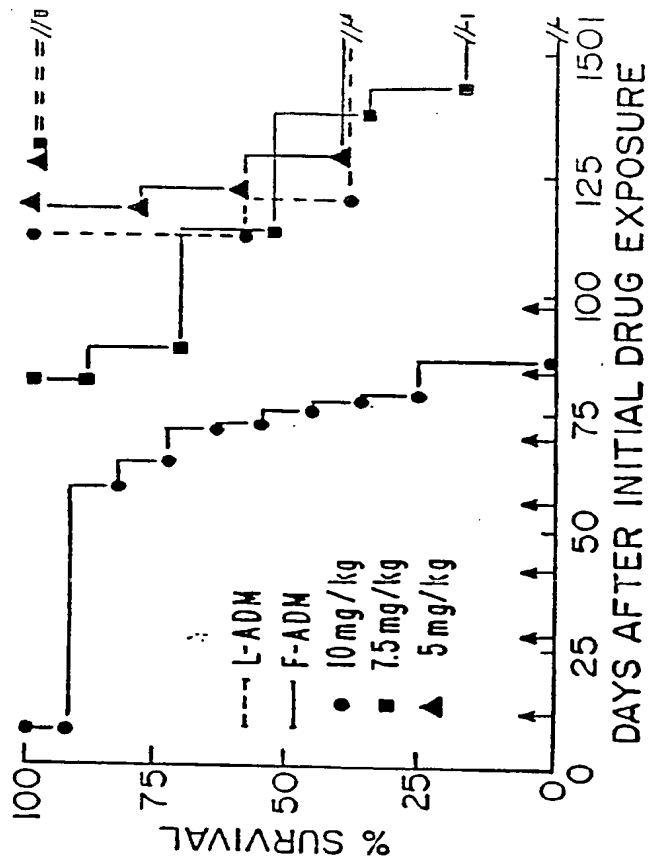
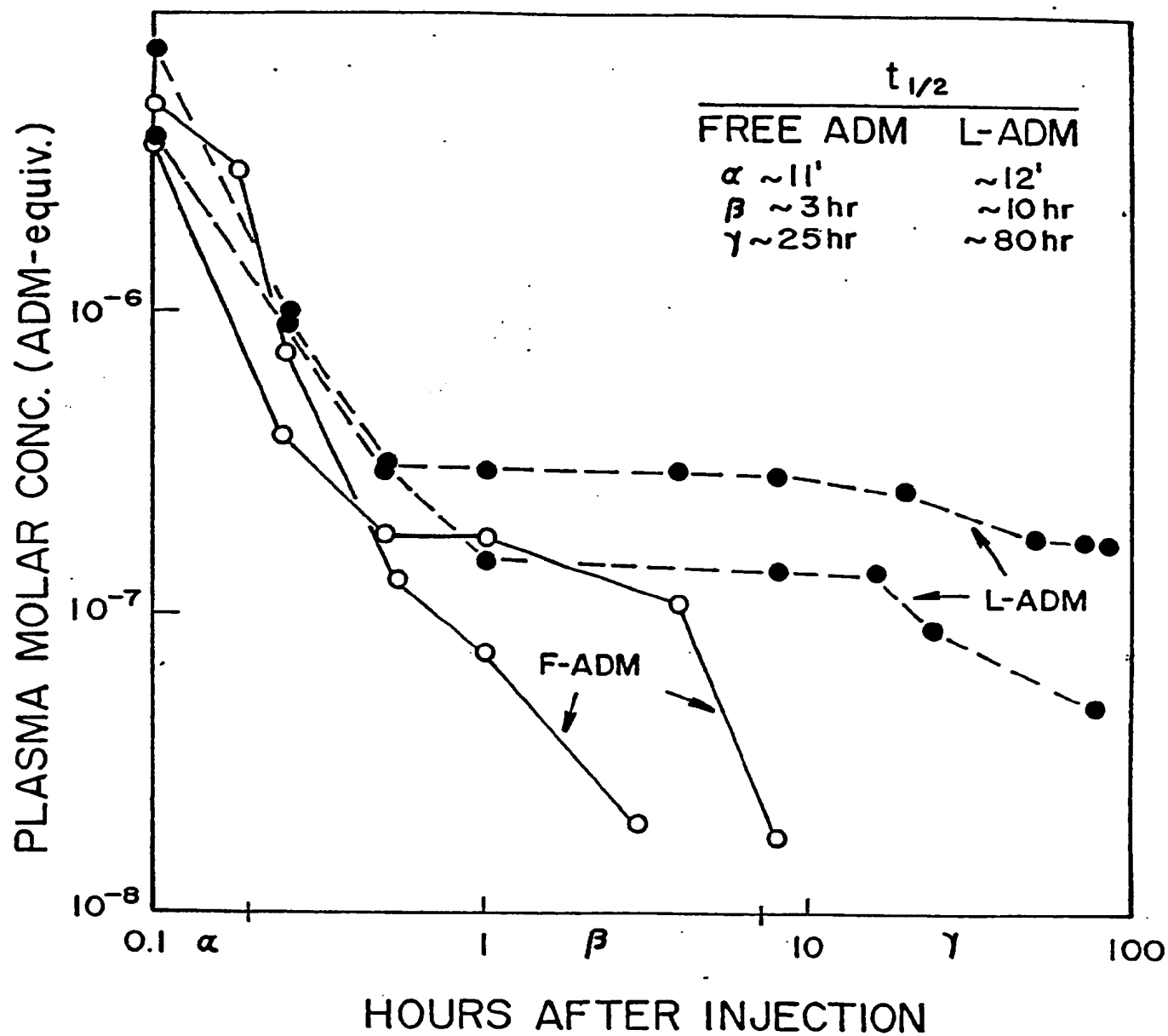


FIG. 8





DOCUMENTS CONSIDERED TO BE RELEVANT			
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.4)
Y	WO-A-8 203 769 (GEORGETOWN UNIVERSITY) * Claims *	1-21	A 61 K 9/50 A 61 K 31/70
A	--- US-A-4 310 506 (J.D. BALDESCHWIELER) * Claims *	1-21	
Y	--- CHEMICAL ABSTRACTS, vol. 98, no. 9, 28th February 1983, page 223, abstract no. 67308y, Columbus, Ohio, US; J.M.C. GUTTERIDGE et al.: "Adriamycin-dependent damage to deoxyribose: a reaction involving iron, hydroxyl and semiquinone free radicals", & FEBS LETT. 1982, 149(2), 228-32 * Whole abstract *	1-6, 12 -14	
Y	--- CHEMICAL ABSTRACTS, vol. 104, no. 17, 28th April 1986, page 34, abstract no. 141870m, Columbus, Ohio, US; J.H. DOROSHOW: "Prevention of doxorubicin-induced killing of MCF-7 human breast cancer cells by oxygen radical scavengers and iron chelating agents", & BIOCHEM. BIOPHYS. RES. COMMUN. 1986, 135(1), 330-5 * Whole abstract *	1-6, 12 -14	C 07 H A 61 K
The present search report has been drawn up for all claims			
Place of search THE HAGUE		Date of completion of the search 17-09-1987	Examiner BERTE M.J.
CATEGORY OF CITED DOCUMENTS			
X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document		T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons & : member of the same patent family, corresponding document	

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DOCUMENTS CONSIDERED TO BE RELEVANT			
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.4)
Y	CHEMICAL ABSTRACTS, vol. 100, no. 3, 16th January 1984, page 25, abstract no. 17331q, Columbus, Ohio, US; D.A. ROWLEY et al.: "DNA damage by superoxide-generating systems in relation to the mechanism of action of the antitumor antibiotic adriamycin", & BIOCHIM. BIOPHYS. ACTA 1983, 761(1), 86-93 * Whole abstract *	1-6, 12-14	
Y	--- CHEMICAL ABSTRACTS, vol. 94, no. 24, 15th June 1981, page 351, abstract no. 197501j, Columbus, Ohio, US; E.H. LAU et al.: "Liposome-encapsulated desferrioxamine in experimental iron overload", & BR. J. HAEMATOL. 1981, 47(4), 505-18 * Whole abstract * -----	1, 5	
The present search report has been drawn up for all claims			TECHNICAL FIELDS SEARCHED (Int. Cl.4)
Place of search THE HAGUE		Date of completion of the search 17-09-1987	Examiner BERTE M.J.
CATEGORY OF CITED DOCUMENTS X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document T : theory or principle underlying the invention E : earlier patent document, but published on. or after the filing date D : document cited in the application L : document cited for other reasons & : member of the same patent family, corresponding document			

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